Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical and Biophysical Research Communications 341 (2006) 51–56
BBRC
www.elsevier.com/locate/ybbrc

Regulation of superoxide stress in *Pseudomonas putida* KT2440 is different from the SoxR paradigm in *Escherichia coli*

Woojun Park [a,b,*,1], Samuel Peña-Llopis [c,1], Yunho Lee [a], Bruce Demple [c]

[a] *Division of Environmental Science and Ecological Engineering, Korea University, Anam-Dong 5Ga, Seoul 136-701, Republic of Korea*
[b] *Environmental Biotechnology National Core Research Center, Gyeongsang National University, Jinju 660-701, Republic of Korea*
[c] *Department of Genetics and Complex Diseases, Harvard School of Public Health, Boston, MA 02115, USA*

Received 15 December 2005
Available online 6 January 2006

Abstract

In *Escherichia coli*, the *SoxR* regulon orchestrates genes for defense against certain types of oxidative stress through the SoxR-regulated synthesis of the SoxS transcription activator. The *Pseudomonas putida* genome did not reveal a clear *soxS* homolog. The *P. putida* SoxR protein appears to be functional: its expression in an *E. coli* Δ*soxR* strain restored the paraquat inducibility of *soxS*. Of nine candidate *P. putida* oxidative stress genes, which are known to be SoxR regulon in *E. coli*, tested for response to superoxide or nitric oxide, *fumC-1*, *sodA*, *zwf-1*, and particularly *fpr*, encoding ferredoxin:$NADP^+$ reductase, were induced, all independent of *P. putida soxR*. Disruption of the *fpr* and *finR*, a regulatory protein that is required for paraquat-dependent expression of the *fpr*, resulted in more oxidative stress sensitivity. However, a *P. putida soxR*-deletion strain had normal resistance to the superoxide-generating agent paraquat. The data presented here show that the genetic responses to superoxide stress in *P. putida* differ markedly from those seen in *E. coli* and *Salmonella*, and the role of *P. putida soxR* remains to be established.

Keywords: Paraquat; Nitric oxide; Ferredoxin reductase; LysR-type transcriptional factor; Oxidative stress; Biodegradation

Pollutant-degrading bacteria may experience oxidative stress, both as a direct effect of the pollutants themselves, and from intermediates generated during biodegradation processes [1,2]. Many redox-cycling pollutants generate superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2), which in the presence of redox-active metals (e.g., copper, iron), can produce hydroxyl radicals inside bacterial cells [2]. These reactive oxygen species (ROS) can be damaging for bacterial cells and decrease the survival rate of indigenous or introduced microorganisms for bioremediation. Regulated adaptive responses of microorganisms to oxidative stress have been extensively studied using *Escherichia coli* and *Salmonella enterica* as model microorganisms [3–7]. However, the oxidative stress response of common soil microorganisms has been poorly characterized.

The SoxR regulon in *E. coli* plays important roles in protection against superoxide and nitric oxide stresses [5,8–10]. The SoxR regulon is controlled by the SoxR, a MerR-family transcription factor containing redox-active [2Fe-2S] centers. SoxR is activated upon [2Fe-2S] oxidation, and it then induces transcription of the divergently transcribed *soxS* gene [10]. The SoxS protein is the direct transcriptional activator of the individual *SoxR* regulon genes including the manganese superoxide dismutase (*sodA*), the DNA repair enzyme endonuclease IV (*nfo*), fumarase C (*fumC*), and ferredoxin/flavodoxin oxido-reductase (*fpr*), among >60 regulon genes [5,11–13]. *Pseudomonas putida* is a metabolically versatile, saprophytic soil bacterium that has been extensively studied as an experimental model soil microorganism [14,15]. The genome sequence of *P. putida* KT2440 has been recently completed [16] and provides a new framework for such an analysis [17]. The genome of *P. putida* KT2440 reveals that it possesses a *soxR* homolog, but there is no obvious *soxS*

* Corresponding author. Fax: +82 2 953 0737.
E-mail address: wpark@korea.ac.kr (W. Park).
[1] These authors contributed equally to this work.

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.12.142

homolog. In this work, we found that *P. putida* SoxR appeared to be functional, based on trans-complementation experiments with *E. coli*. However, none of several oxidative stress-inducible genes was dependent on the *P. putida* SoxR. Although the *fpr* gene was not regulated by SoxR, it was induced dependent on FinR, a novel LysR-type transcription factor encoded by a gene adjacent to *fpr*. Thus, regulation by superoxide stress in *P. putida* KT2440 may be quite different from the SoxR mechanism of *E. coli*.

Materials and methods

Bacterial strains and culture conditions. The bacterial strains used in this study are listed in Table 1. Bacterial cultures were grown at 37 °C for *E. coli* and at 30 °C for *Pseudomonas* strains in Luria–Bertani (LB) broth with vigorous aeration by shaking at 250 rpm. Kanamycin (100 μg/ml) or tetracyline (15 μg/ml) was added as necessary. Paraquat (PQ), menadione (MD), and spermine-diazeniumdiolate (spermine-NONOate) were added at the final concentrations described in the figure legends. Rifampicin (200 μg/ml) was added to stop further transcription.

DNA and plasmid manipulation. PCR and cloning for vector construction were performed as described by Ausubel et al. [18]. The primers used in this study are listed in Table 2. The 713-bp fragment of *soxR* region, amplified using a set of primers (PsoxRS-3/Psox-P2), were cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA), generating pWP100. The plasmid was introduced into *E. coli* INV-αF′ competent cells (Invitrogen). Both directions of the insert fragment were sequenced, and all sequencing was completed at Dana-Farber/Harvard Cancer Center. The verified plasmid was subcloned into *E. coli* EH46 strain (Δ*soxRS*, *soxS*′::*lacZ*) creating EH46 (pWP100).

Construction of P. putida KT2440 soxR mutant. The suicide transcriptional fusion vector, pVIK112 [19], was used for constructing mutation in the *soxR* gene of *P. putida*. Primer pair SoxR-D1/SoxR-D2 was designed to target an internal region of the *soxR* gene. Construction and verification of the *soxR* mutant was performed using a previously described procedure [20]. Construction of the *finR* and *fpr* mutants was previously described [20].

Electrophoretic mobility shift assay. Electrophoretic mobility shift assay (EMSA) was performed using a previously described procedure [21], except that the putative *P. putida* SoxR-binding site was amplified with the Psox-P1/Psox-P2 primer pair, which yields a 255-bp fragment.

Northern blot analysis. Northern blot analysis was performed as described previously [20]. The primer pairs used in Northern blot analysis are listed in Table 2. The primer pairs were amplified by PCR for preparing the specific probes for *acnA*, *fpr*, *fumC-1*, *fur*, *ribA*, *rimK*, *sodA*, *tolC*, and *zwf-1*, respectively.

Quantitative real-time PCR. Total RNA was treated with RNase-free DNase I (Ambion) to eliminate contaminating DNA and reverse transcribed with the Reverse Transcription System (Promega). Quantitative real-time PCR (QRT-PCR) was performed using Brilliant SYBR Green Master Mix QPCR (Stratagene) in an iCycler real-time thermocycler (Bio-Rad). Standard curves were made with different concentrations of the DNA probes used for Northern blots.

Results and discussion

Sequence analysis of a SoxR homolog in P. putida KT2440

The genome of *P. putida* KT2440 encodes a SoxR homolog with 62% amino acid identity with SoxR of *E. coli*, and 56% identity with the *P. aeruginosa* PAO1 protein. All have four conserved cysteine residues corresponding to those required for anchoring the [2Fe-2S] centers of *E. coli* SoxR (positions 119, 122, 124, and 130). The head-to-head arrangement of the *soxR* and *soxS* genes was seen only in *E. coli* and *S. enterica*. The SoxR and SoxS in these two species function similarly [4], while a clear *soxS* homolog was not found in the other genomes analyzed. The NCBI annotation of the *soxR* region in *P. putida* KT2440 is shown as the upper diagram in Fig. 1A, with the proposed *soxR*-proximal gene of unknown function denoted as PP2061. PP2061 does not have a homolog in the NCBI database. The lower diagram in Fig. 1A indicates an alternative *soxR*-proximal gene *hyp* oriented opposite to PP2061. No *hyp* homolog is present in the NCBI database, but the orientation of *hyp* relative to *soxR* and the intergenic separation are very similar to the arrangement of the *E. coli* soxRS locus. Interestingly, the putative 18-bp SoxR binding site of *P. putida* KT2440 is present upstream of the *soxR* gene and differs by only three bp from that of *E. coli* SoxR. The sequence analysis implied that SoxR of *P. putida* KT2440 might function similarly to *E. coli* protein in redox sensing, DNA binding, and transcriptional activation of the neighboring gene.

Table 1
Bacterial strains and plasmids used in this study

Bacterial strain/plasmid	Description	Reference
Strains		
E. coli INV-αF′	$F'^{endA1recA1hsdR17}(r_k^-, m_k^+)$ *supE44 thi-1 gyrA96 relA1 ϕ80lacZM15 ΔlacZYA-argF) U169*	Invitrogen
E. coli GC4468	*E. coli* K12 *rpsL thi soxR*$^+$ *soxS*$^+$	[31]
E. coli EH46	DJ901 (derived from GC4468 by introduction of the *soxRS901* deletion), lysogenized with λEH40 carrying a *soxS*′::*lacZ* transcriptional fusion)	[31]
E. coli EC100D *pir*-116	Tra$^-$ R6K strain	Epicentre
E. coli S17-1 *λpir*	Tra$^+$ R6K strain	[19]
P. putida KT2440	TOL plasmid-cured derivative of *P. putida* mt-2	[16]
P. putida KT2440Tc	TOL plasmid-cured derivative of *P. putida* mt-2 with mini-Tn5 insertion of Tet^R	[32]
P. putida KT2440-SoxR	*soxR*$^-$ mutant, derivative of *P. putida* KT2440, insertion of pHU2	This study
P. putida KT2440-FinR	*finR*$^-$ mutant, insertion of pVIK-finR into *P. putida* KT2440	[20]
P. putida KT2440-Fpr	*fpr*$^-$ mutant, insertion of pVIK-KO1 into *P. putida* KT2440	[20]
Plasmids		
pVIK112	R6K *oriV*, suicide vector, *lacZ* fusion.	[19]
pWP100	pCR2.1-TOPO vector containing the *P. putida soxR* region	This study
pHU1	Internal *soxR* fragment in pCR2.1-TOPO	This study

Table 2
Primers used in this study

Name	Sequence 5′–3′	mer
acnA Pp-hA	GTT GCC GCC TTC TTC ACC	18
acnA Pp-hS	GAC CAC CGA CCA CAT TTC CC	20
fpr Pp-hA	GCC GGT GCT CAG CAG GTA C	19
fpr Pp-hS	CCA TCG CTT CGC CAA ACT G	19
fumC1 Pp-hA	GAT GTC ATT GCT GCT CTG G	19
fumC1 Pp-hS	GTT CAT TCG TGC CTT GTT G	19
fur Pp-hA	CTG GGT CAG CAC GCG ATA G	19
fur Pp-hS	GCA AAG CCG GTC TCA AGG	18
Psox-P1	CGC GGT ACC GAT CAG CCC CTT GGT TTC GTA G	31
Psox-P2	CGC GAA TTC CGT GCG TCG TGG CTT TGG GTA A	31
Psox-P2	CGC GAA TTC CGT GCG TCG TGG CTT TGG GTA A	31
PsoxRS-3	ACA GGG TTA GGG CGG TGA TTC GTC	24
pVIK-R	ACC ATG GTC ATA GCT GTT TCC TG	23
ribA Pp-hA	CTC GCC CTG GTG TTC GTT GC	20
ribA Pp-hS	CAA GTC GCT GCG CCT GAT G	19
rimK Pp-hA	CGG TGA TGC GCT CTT CTG G	19
rimK Pp-hS	CAT TGG CGT GGT GCT GTG C	19
sodA Pp-hA	CAG GGC TTC AAG GTA ACG AC	20
sodA Pp-hS	GAG TAG CGG CAA CCA GGA C	19
SoxR-D1	CGC GAG CTC GCA GCG GCG TGG CGG TGT CT	29
SoxR-D2	CGC GGT ACC TGC GGG CAT TCA GGT CTT C	28
tolC Pp-hA	GGT GCG TCC TAC TTC ATT CG	20
tolC Pp-hS	GGT GGC TAC TAC CCT TCC G	19
zwf1 Pp-hA	CAG CAG CGA ACG GGT CATC	19
zwf1 Pp-hS	CTG TAC CAG CAG GCG GAG AAC	21

Restriction enzyme sites, used for cloning purposes, are underlined.

Complementation of an E. coli soxR-null mutant with P. putida KT2440 SoxR

We tested the functionality of *P. putida* SoxR initially by cross-complementation of an *E. coli* Δ*soxR* mutant. *P. putida* SoxR was amplified and subcloned into the pCR2.1-TOPO vector. After verification of the sequences, the resulting vector (pWP100) was transformed into *E. coli* strain EH46 (Δ*soxRS*, *soxS ′::lacZ*). Northern blot analysis was performed to confirm the expression of SoxR in the *E. coli* strains using a *P. putida* SoxR probe (Fig. 1B). It appeared that *P. putida* SoxR was expressed from the plasmid with or without PQ treatment of the bacteria. PQ treatment strongly elevated the *P. putida* SoxR-dependent transcription of the *E. coli soxS* promoter about sixfold (Fig. 1C). The PQ-responsiveness of *soxS* transcription dependent on the *P. putida soxR* plasmid indicates that the *Pseudomonas* homolog undergoes redox regulation, probably through [2Fe-2S] centers.

Binding of E. coli SoxR to the putative SoxR binding site of P. putida KT2440 in vitro

Only one putative SoxR binding-like site was found in the *P. putida* KT2440 genome by searching for sequence motifs (CCTMAAGTTWRSTTGAGS), which is the intergenic region between *soxR* and a hypothetical gene (Fig. 1A). However, this SoxR binding-like site is present in three loci (upstream of PA2274, PA3718, and *mexG*) in the genome of *P. aeruginosa* PAO1 [22]. There are no such homologs or any other likely SoxR binding sites found in the genome of *P. putida* KT2440. This *P. putida* SoxR binding-like site can be bound by *E. coli* SoxR in vitro (Fig. 2), but several attempts by Northern blot and primer extension analysis failed to show increased transcription of the hypothetical gene upstream of *soxR* under conditions known to activate *E. coli soxS* expression (data not shown). The *P. putida soxR* gene itself was also not induced under any conditions tested in Northern blotting experiments (data not shown). However, in vivo activity of the *P. putida soxR* promoter fused to the *lacZ* gene was detected (data not shown), and the analysis indicated that the gene is negatively autoregulated (12.9 Miller units in a $soxR^+$ strain vs. 40 Miller units in a $soxR^-$ strain), as is the case with *E. coli soxR* [23]. The *P. putida* KT2440 *soxR* gene was disrupted by Campbell-type, single-crossover homologous recombination [19] using the suicide plasmid pHU2 (containing an internal region of *P. putida soxR*). The growth rate of this *soxR* mutant strain was the same as $soxR^+$ *P. putida* in both rich and minimal medium, and both strains showed similar growth inhibition by added PQ (data not shown). Further experimentation is still needed to determine whether *P. putida soxR* is involved in oxidative stress or other responses in that organism.

Induction of superoxide stress genes, that are known to be SoxR regulon in E. coli, in P. putida KT2440

To gain further insight into oxidative stress responses in *P. putida*, we examined the expression of nine genes (*acnA,*

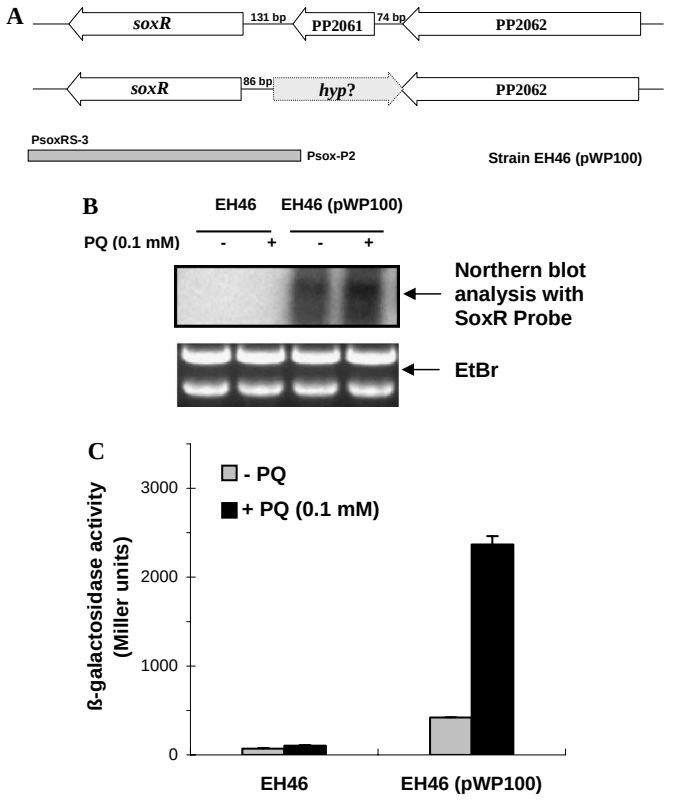

Fig. 1. Complementation of *E. coli* soxR-null mutant with *P. putida* KT2440 SoxR. (A) Diagram shows the genetic organization of the soxR and hyp (hypothetical) genes in *P. putida* KT2440, and the fragment amplified with PsoxRS-3/Psox-P2 primer pair is indicated (hyp: hypothetical protein; numbers between two genes are the length of the intergenic region between the *soxR* gene and the upstream gene. (B) Northern blot analysis using the *soxR* gene probe generated by PCR using SoxR-D1/SoxR-D2 primer pair. The ethidium bromide-stained (EtBr) gel prior to blotting is also shown, which demonstrates consistent loading. The growth rate of the two strains EH46, EH46(pWP100) was similar in LB medium (data now shown). (C) The level of *soxS′::lacZ* reporter expression, measured by β-galactosidase activity, is shown for each strain. The bars represent means and standard deviations of three determinations.

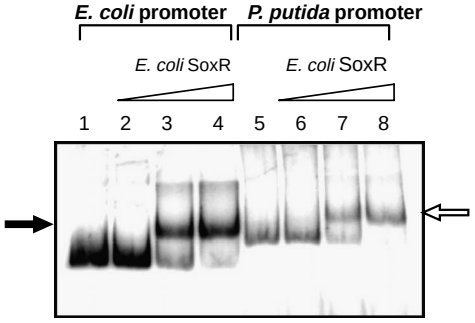

Fig. 2. Binding of *E. coli* SoxR to the putative SoxR binding site of *P. putida* KT2440 in vitro. Protein–DNA binding was measured by EMSA. The concentration of purified *E. coli* SoxR increases from lane 1 to lane 4 (0 nM in lanes 1, 5; 1 nM in lanes 2, 6; 10 nM in lanes 3, 7; 20 nM in lanes 4, 8). Binding of purified *E. coli* SoxR to the fragment (180 bp) containing the *E. coli* SoxR binding site and the fragment (255 bp) containing the putative *P. putida* SoxR binding site is indicated by the arrows (filled and open, respectively).

fpr, fumC-1, fur, ribA, rimK, sodA, tolC, and *zwf-1*) by Northern blot and QRT-PCR. The *E. coli* counterparts of these genes are known to be involved in the SoxR-mediated oxidative stress defense [5]. Only *fpr*, *fumC-1*, *sodA*, and *zwf-1* genes were induced by superoxide and nitric oxide in Northern blotting analysis (Fig. 3A). The expression of the *P. putida acnA*, *fur*, *rimK*, and *tolC* genes, which are twofold induced by PQ in *E. coli* [24], was not induced in *P. putida* by any of the agents tested in Northern blot analysis (data now shown). Since the hybridization of some blots with *acnA*, *ribA*, and *rimK* gave rather weak signals, their expression levels were confirmed by QRT-PCR (Table 3). PQ-induced expression of oxidative stress genes determined by QRT-PCR was highly correlated with Northern blotting ($r = 0.961$, $P < 0.001$). The QRT-PCR data, in the presence of PQ (1 mM) for 10 min, demonstrated that the expression of *acnA*, *ribA*, and *rimK* genes are not induced, although there is a slight increase of expression of the *acnA* gene (Table 3). The highest *fpr* expression was detected within 1 min for menadione or nitric oxide, but the PQ-dependent induction was higher at 10 min. The expression of class II fumarate hydratase (*fumC-1*) and manganese superoxide dismutase (*sodA*) genes required at least 10 min of exposure to superoxide (Fig. 3A). Conversely, the highest expression of glucose

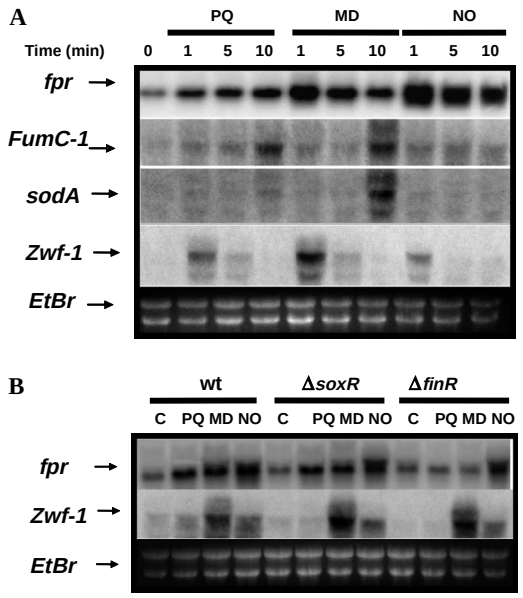

Fig. 3. Induction of the oxidative stress genes in response to oxidative and nitrosative stress in *P. putida* KT2440. (A) Northern blot analysis of the *fpr*, *fumC-1*, *sodA*, and *zwf-1* gene expression is shown. Total mRNA was extracted at 1, 5, or 10 min after treatment of cells with 1 mM PQ (PQ), 0.5 mM menadione (MD), and 0.1 mM spermine-NONOate (NO). The ethidium bromide-stained (EtBr) gel prior to blotting demonstrated consistent loading in all lanes. (B) Induction of *fpr* and *zwf-1* genes by oxidative or nitrosative stress in *P. putida* KT2440 wild-type, a *soxR* mutant (Δ*soxR*), and a *finR* mutant (Δ*finR*). A different incubation time was used for each reagent to maximize *fpr* expression: 1 mM paraquat (PQ), 10 min; 0.5 mM menadione (MD), 1 min; 0.1 mM spermine-NONOate (NO), 1 min. The experiment was repeated at least three times with similar results.

Table 3
Comparison of QRT-PCR and Northern blotting on the relative mRNA abundance of oxidative stress genes in *P. putida* upon exposure to 1 mM PQ for 10 min

Gene	Femtograms of mRNA		PQ/ctrl ratio for QRT-PCR		PQ/ctrl ratio for Northern		r
	Controls	Paraquat	Average	95% CI	Average	95% CI	
acnA	0.49 ± 0.08	0.61 ± 0.13	1.26 ± 0.29	0.80–1.71	n.d.	—	—
fpr	4.13 ± 1.40	11.86 ± 1.70**	2.87 ± 0.34	2.34–3.41	2.59 ± 0.06	2.49–2.69	0.974
fumC-1	2.81 ± 0.51	7.17 ± 1.23*	2.55 ± 0.36	1.99–3.12	1.72 ± 0.29	1.25–2.18	0.840
ribA	4.20 ± 0.51	4.02 ± 0.09	0.97 ± 0.11	0.79–1.14	0.76 ± 0.06	0.67–0.85	0.960
rimK	2.02 ± 0.15	1.77 ± 0.19	0.88 ± 0.09	0.74–1.01	0.85 ± 0.14	0.64–1.07	0.908
sodA	0.67 ± 0.19	1.26 ± 0.17*	1.88 ± 0.20	1.56–2.21	1.51 ± 0.44	0.81–2.22	0.858
tolC	70.0 ± 7.9	68.3 ± 5.2	0.98 ± 0.06	0.88–1.07	0.89 ± 0.05	0.81–0.97	0.912
zwf-1	2.53 ± 0.64	2.57 ± 0.28	0.97 ± 0.15	0.74–1.21	0.85 ± 0.15	0.61–1.09	0.917

r, the Pearson correlation coefficient; ctrl, control; PQ, paraquat.
* $P < 0.01$.
** $P < 0.001$ for an independent-samples *T* test; n.d., not detected.

6-phosphate 1-dehydrogenase (*zwf-1*) gene upon oxidative and nitrosative stress was within 1 min and decreased rapidly (Fig. 3A). The QRT-PCR data indicated that the *fpr*, *fumC-1*, and *sodA* genes were highly induced (2.9-, 2.6-, and 1.9-fold, respectively) in the presence of PQ (1 mM) for 10 min (Table 3). However, consistent with the Northern blot analysis where the *zwf-1* gene was induced within 1 min and decreased rapidly, the *zwf-1* gene was not induced in the 10 min exposure of the QRT-PCR analysis (Table 3). Interestingly, the induction of *fpr*, *fumC-1*, *sodA*, and *zwf-1* genes by oxidative and nitrosative stress was independent of *P. putida* SoxR, because a strain of *P. putida* defective in SoxR behaved similarly to the wild type when exposed to PQ, menadione or nitric oxide (Fig. 3B, data now shown for the *fumC-1* and *sodA* genes).

Previously, we have shown that FinR, a LysR-type transcriptional regulator, is required for induction of the *fpr* gene in response to PQ or menadione [20]. Although oxidative stress-induced expression of *fpr* in *E. coli* is dependent on *soxR* [25,26], in *P. putida fpr* was still inducible by PQ, menadione, and nitric oxide in a Δ*soxR* strain (Fig. 3B). However, in a *P. putida* strain deleted for the FinR protein, *fpr* induction in response to PQ or menadione was lost (Fig. 3B). Interestingly, FinR is not required for the *fpr* response to nitric oxide in *P. putida* (Fig. 3B), which suggests the existence of additional regulators. Furthermore, the induction of *fumC-1*, *sodA*, and *zwf-1* by superoxide or nitric oxide was not dependent of SoxR or FinR (Fig. 3B, data now shown), indicating the involvement of other regulators. Exponentially grown (~10^7 cells) aliquots of each strain were spotted onto LB agar containing PQ. The *fpr* and *finR* mutants could not grow in the presence of PQ (0.2 mM) after three days incubation (Fig. 4). However, at lower concentration of PQ (0.04 mM), the *finR* mutant could grow after two days incubation (Fig. 4). Growth inhibition of the *soxR* mutant of *P. putida* KT2440 by PQ was not severe in agar-plate assay (Fig. 4).

While bacterial transcriptional factors that respond to oxidative stress have been extensively characterized in *E. coli* and *S. enterica* [4,5,16], much less has been learned

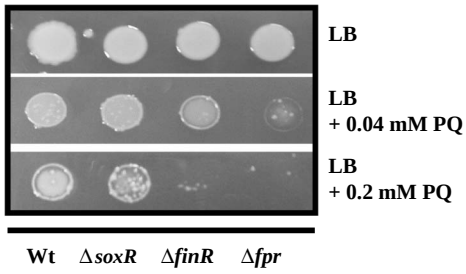

Fig. 4. Phenotypic assays of wild-type and mutant strains of *Pseudomonas putida* KT2440. Growth of all tested strains on LB agar plate containing PQ. Cells (10^7) in exponential growth were spotted on LB agar with and without Paraquat (PQ), at the final concentrations shown in the figure.

for the *Pseudomonas* species, including the important model soil microorganism *P. putida* KT2440. We focused initially on SoxR, which in *E. coli* and *S. enterica* governs responses to superoxide-generating agents, such as PQ and menadione, and to nitric oxide [5]. For the several genes examined, *P. putida soxR* did not seem to be involved in the response to superoxide or nitric oxide. The *P. putida* protein is clearly functional and can be redox-regulated, as demonstrated when it was expressed in *E. coli*, which highlights the question of what genes in *P. putida* might be SoxR-controlled. In contrast with the results found here, Kobayahsi and Tagawa [27] have shown that the gene of unknown function located near SoxR (in the same position of *soxS*) in *P. aeruginosa* 3080 strain is controlled by SoxR. We cannot exclude the possibility that a completely new oxidative defense system may have evolved in *Pseudomonas* species, thus rendering SoxR-dependent system obsolete or taking over the control of genes that were formerly *SoxR* regulon. This hypothesis is supported by the fact that *Pseudomonas* species have more complex regulatory system than *E. coli* system [3,7,28–30] and the genome of *Pseudomonas syringae* pv. tomato str. DC3000 does not have a SoxR homolog. Current efforts are seeking to identify *soxR*-regulated genes in *P. putida* directly. Our data suggest that superoxide stress responses in *P. putida* KT2440 differ in their regulation from the systems of *E. coli*. These studies

will enhance the understanding of the physiology and ecology of soil microorganisms in bioremediation processes where oxidative stress is an important challenge.

Acknowledgments

S.P.-L. was supported by a postdoctoral fellowship from the Valencian Government (Generalitat Valenciana) in Spain. This work was supported by NCI, National Institutes of Health Grant CA37831 to B.D. and a NCRC (National Core Research Center) Grant (R15-2003-002-01002-0), a grant (R0503441) from BioGreen21 program, Rural Development Administration, and Ministry of Education and Human Resource Development (Grant R0508751), Republic of Korea to W.P.

References

[1] W. Park, C.O. Jeon, H. Cadillo, C. DeRito, E.L. Madsen, Survival of naphthalene-degrading *Pseudomonas putida* NCIB 9816-4 in naphthalene-amended soils: toxicity of naphthalene and its metabolites, Appl. Microbiol. Biotechnol. 64 (2004) 429–435.

[2] N.A. Schewigert, J.B. Zehnder, R.I.L. Eggen, Chemical properties of catechols and their molecular modes of toxic action in cells, from microorganisms to mammals, Environ. Microbiol. 3 (2001) 81–91.

[3] B. Demple, Redox signaling and gene control in the *Escherichia coli soxRS* oxidative stress regulon—a review, Gene 179 (1996) 53–57.

[4] P.J. Pomposiello, B. Demple, Identification of SoxS-regulated genes in *Salmonella enterica* serovar typhimurium, J. Bacteriol. 182 (2000) 23–29.

[5] P.J. Pomposiello, B. Demple, Redox-operated genetic switches: the SoxR and OxyR transcription factors, Trends Biotechnol. 19 (2001) 109–114.

[6] G. Storz, J.A. Imlay, Oxidative stress, Curr. Opin. Microbiol. 2 (1999) 188–194.

[7] M. Zheng, X. Wang, L.J. Templeton, D.R. Smulski, R.A. LaRossa, G. Storz, DNA microarray-mediated transcriptional profiling of the *Escherichia coli* response to hydrogen peroxide, J. Bacteriol. 183 (2001) 4562–4570.

[8] H. Ding, B. Demple, Direct nitric oxide signal transduction via nitrosylation of iron–sulfur centers in the SoxR transcription activator, Proc. Natl. Acad. Sci. USA 97 (2000) 5146–5150.

[9] E. Hidalgo, B. Demple, Spacing of promoter elements regulates the basal expression of the *soxS* gene and converts SoxR from a transcriptional activator into a repressor, EMBO J. 16 (1997) 1056–1065.

[10] E. Hidalgo, H. Ding, B. Demple, Redox signal transduction: mutations shifting [2Fe-2S] centers of the SoxR sensor-regulator to the oxidized form, Cell 88 (1997) 121–129.

[11] P. Mukhopadhyay, M. Zheng, L.A. Bedzyk, R.A. LaRossa, G. Storz, Prominent roles of the NorR and Fur regulators in the *Escherichia coli* transcriptional response to reactive nitrogen species, Proc. Natl. Acad. Sci. USA 101 (2004) 745–750.

[12] R.G. Martin, J.L. Rosner, The AraC transcriptional activators, Curr. Opin. Microbiol. 4 (2001) 132–137.

[13] R.G. Martin, J.L. Rosner, Analysis of microarray data for the *marA, soxS*, and *rob* regulons of *Escherichia coli*, Methods Enzymol. 370 (2003) 278–280.

[14] L.P. Wackett, *Pseudomonas putida*—a versatile biocatalyst, Nat. Biotechnol. 21 (2003) 36–138.

[15] I. Cases, V. de Lorenzo, C.A. Ouzounis, Transcription regulation and environmental adaptation in bacteria, Trends Microbiol. 11 (2003) 248–253.

[16] K.E. Nelson, C. Weinel, I.T. Paulsen, et al., Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440, Environ. Microbiol. 4 (2002) 799–808.

[17] D. Stjepandic, C. Weinel, H. Hilbert, H.L. Koo, F. Diehl, K.E. Nelson, B. Tummler, J.D. Hoheisel, The genome structure of *Pseudomonas putida*: high-resolution mapping and microarray analysis, Environ. Microbiol. 4 (2002) 819–823.

[18] F.M. Ausubel, R. Brent, R. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, K. Struthl, Current Protocols in Molecular Biology, John Wiley & Sons Inc, New York, NY, 1999.

[19] V.S. Kalogeraki, S.C. Winans, Suicide plasmids containing promoterless reporter genes can simultaneously disrupt and create fusions to target genes of diverse bacteria, Gene 188 (1997) 69–75.

[20] Y. Lee, S. Pena-Llopis., Y.-S. Kang, H.-D. Shin, B. Demple, E.L. Madsen, C.O. Jeon, W. Park, Expression analysis of the *fpr* (ferredoxin-$NADP^+$ reductase) gene in *Pseudomonas pustida* KT2440, Biochem. Biophys. Res. Commun. 339 (2006) 1246–1254.

[21] E. Hidalgo, B. Demple, An iron–sulfur center essential for transcriptional activation by the redox-sensing SoxR protein, EMBO J. 13 (1994) 138–146.

[22] M. Palma, J. Zurita, J.A. Ferreras, S. Worgall, D.H. Larone, L. Shi, F. Campagne, L.E. Quadri, *Pseudomonas aeruginosa* SoxR does not conform to the archetypal paradigm for SoxR-dependent regulation of the bacterial oxidative stress adaptive response, Infect. Immun. 73 (2005) 2958–2966.

[23] E. Hidalgo, V. Leautaud, B. Demple, The redox-regulated SoxR protein acts from a single DNA site as a repressor and an allosteric activator, EMBO J. 17 (1998) 2629–2636.

[24] P.J. Pomposiello, M.H. Bennik, B. Demple, Genome-wide transcriptional profiling of the *Escherichia coli* responses to superoxide stress and sodium salicylate, J. Bacteriol. 183 (2001) 3890–3902.

[25] A.R. Krapp, E. Rodriguez, D. Poli, H. Paladini, J.F. Palatnik, N. Carrillo, The Flavoenzyme ferredoxin (flavodoxin)-NADP(H) reductase modulates NADP(H) homeostasis during the *soxRS* response of *Escherichia coli*, J. Bacteriol. 184 (2002) 1474–1480.

[26] S.I. Liochev, A. Hausladen, W.F. Beyer Jr., I. Fridovich, NADPH:Ferredoxin oxidoreductase acts as a Paraquat diaphoras and is a member of the *soxRS* Regulon, Proc. Natl. Acad. Sci. USA 91 (1994) 1328–1331.

[27] K. Kobayashi, S. Tagawa, Activation of SoxR-dependent transcription in *Pseudomonas aeruginosa*, J. Biochem. 136 (2004) 607–615.

[28] M.A. Martinez-Bueno, R. Tobes, M. Rey, J.L. Ramos, Detection of multiple extracytoplasmic function (ECF) sigma factors in the genome of *Pseudomonas putida* KT2440 and their counterparts in *Pseudomonas aeruginosa* PA01, Environ. Microbiol. 4 (2002) 842–855.

[29] M.A. Ramos-Diaz, J.L. Ramos, Combined physical and genetic map of the *Pseudomonas putida* KT2440 chromosome, J. Bacteriol. 180 (1998) 6352–6363.

[30] V. Venturi, Control of *rpoS* transcription in *Escherichia coli* and *Pseudomonas*: why so different? Mol. Microbiol. 49 (2003) 1–9.

[31] J.T. Greenberg, P. Monach, J.H. Chou, P.D. Josephy, B. Demple, Positive control of a global antioxidant defense regulon activated by superoxide-generating agents in *Escherichia coli*, Proc. Natl. Acad. Sci. USA 87 (1990) 6181–6185.

[32] B. Normander, B.B. Christensen, S. Molin, N. Kroer, Effect of bacterial distribution and activity on conjugal gene transfer on the phylloplane of the bush bean (*Phaseolus vulgaris*), Appl. Environ. Microbiol. 64 (1998) 1902–1909.